

## **Novel Therapeutics for HUS**

### **Tom Obrig, Ph.D.**

The progression of disease after the ingestion of food contaminated with enterohemorrhagic *E. coli* (EHEC) becomes apparent with the onset of diarrhea, abdominal pain, and vomiting approximately three days post-infection. Two days later, diarrhea becomes bloody in 90% of cases. After 4 to 5 days of enterohemorrhagic colitis, the diarrhea resolves spontaneously in 85-90% of cases, but progresses to acute renal failure and the hemolytic uremic syndrome (HUS) in 10-15% of cases. The timeline of disease progression indicates a window of opportunity to intervene during the systemic toxemia phase of disease from day 2.5 to day 7 post-infection, when the bacterial Shiga toxin (Stx2) and lipopolysaccharide (LPS) drive the transition to bloody diarrhea and the risk of HUS.

The observed pathology during the window of opportunity includes host inflammation and coagulation/thrombosis which become possible therapeutic targets in control of the host response. Another potential target is the blocking of toxin receptors. Employment of a combination of these approaches is also possible.

The primary virulence determinants of EHEC impact host cells by different mechanisms. The potent Stx2 binds selectively to glycosphingolipid (Gb3) expressed on the surface of host cells and activates the stress response, inhibits protein synthesis, induces cytokines, and initiates apoptosis. LPS binds TLR4, induces cytokines/chemokines, and magnifies the Stx2 response by inducing the expression of additional Gb3 on target cells.

Adenosine is an anti-inflammatory compound of interest in these studies. Adenosine is normally produced by damaged host cells in many situations. However, newer synthetic analogs of adenosine may offer pharmacological benefits. Adenosine works by binding to and activating specific G protein-coupled receptors on certain cell types to activate a cyclase that generates cyclic AMP (cAMP). Thus, adenosine is a high affinity agonist of the adenosine A<sub>2A</sub> receptor for cAMP production and cAMP indirectly blocks adhesion of monocytes to the vascular endothelium as part of its anti-inflammatory function.

The study of anti-inflammatory compounds necessitated the development of a mouse model of kidney inflammation. Injection of mice with a lethal dose of Stx2 (200 ng/kg), with and without a sublethal dose of LPS (300 ug/kg), was followed temporally by euthanasia, removal of kidneys, and analysis of inflammatory cells, cytokines/chemokines, and selectins/integrins. Renal mRNA was analyzed by microarray, RNase protection assays, and real-time rt-PCR, while renal protein was analyzed by ELISA, western blot and immunohistochemistry. The following temporal events were observed. When Stx2 was applied alone, serum creatinine levels increased steadily reaching a three-fold increase over 84 hours. LPS alone induced Gb3 on renal vascular endothelium. When both LPS and Stx2 were employed, a unique series of events occurred that included induction of renal chemokines and cytokines that attracted monocytes and neutrophils into the kidney, leading to increased adherence of inflammatory cells to endothelia, the activation of platelets, coagulation, thrombosis, endothelial damage, and other manifestations of HUS analogous to those seen in patients. Monocyte/macrophage recruitment to the kidney medulla and cortex of Stx2/LPS-treated mice showed a steady 5 to 10-fold increase over the course of 72 hours. The chemokines for monocyte chemotaxis in mice include MIP-1 $\alpha$  / CCL3, MCP-1 / JE / CCL2, and RANTES / CCL5, which can be identified using immunostaining of tissue sections, revealing intense expression and vascular damage surrounding monocytes when Stx2 and LPS are co-administered.

The ability of the adenosine A<sub>2A</sub> agonist ATL to inhibit the early events in the Stx2/LPS response was assessed using ELISA assay for renal MIP-1 $\alpha$ , MCP-1/JE, RANTES, and monocyte/macrophage recruitment, in the presence and absence of ATL146e at 10ug/kg every 6 hours. Significant differences were detected in the induction of all markers, and the peak cytokine/chemokine responses seen at 2-12 hours were reduced to approximately 60%. In addition, macrophage/monocyte recruitment into the kidney was reduced from a maximum of ~55 cells per field of view to ~12 at 72 hours. Similarly, the effect of ATL146e on renal TNF- $\alpha$  was significant, with treated animals showing significantly reduced expression until 72 hours, when levels returned to baseline in treated and control animals, a pattern also seen in renal IL-1 $\beta$  measurements.

Platelets are activated in HUS due, in part, to the action of Stx2 on the endothelium, which results in the expression and release of chemokines such as SDF-1 $\alpha$  and SDF-1 $\beta$  that are co-activators of platelets. The effects of Stx2, LPS, and ATL-146e on the release of SDF-1 $\alpha$  and SDF-1 $\beta$  were measured using human microvascular endothelial cells (HMEC). Platelet aggregation in vitro is rapidly activated over a period of 5 seconds. In the mouse model, ATL146e reduces the Stx2-dependent peak renal platelet count by 90%. The mechanism of ATL146e action, therefore, appears to be through inhibition of chemokine induction at the endothelial level, and by inhibiting the release of cytokines by monocytes in the kidney.

The effect of another longer acting adenosine A<sub>2A</sub> agonist, ATL303 administered i.p. every 12 hours for 7 days, was tested in a series of preliminary experiments. When ATL303 was administered to Stx2 /LPS treated mice, the time to death was extended in a dose-dependent manner from 2 days (10ug/kg) to >12 days (20ug/kg).

In conclusion:

- A<sub>2A</sub> Receptor agonists inhibit LPS/Stx2-induction of renal chemokines for monocyte migration into kidney of mice.
- A<sub>2A</sub> Receptor agonists inhibit Stx2-induction of renal cytokines produced by monocytes.
- A<sub>2A</sub> Receptor agonists inhibit Stx2-induced accumulation of monocytes and platelets in kidneys of mice.
- A<sub>2A</sub> Receptor agonists reduce Stx2-induced lethality in mice.

RESEARCH NEEDS:

- Identification of lead therapeutic candidates for preclinical and clinical development.